Microbial contaminants in wild harvested and traded edible long-horned grasshopper, *Ruspolia differens* (Orthoptera: Tettigoniidae) in Uganda

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Abstract

This study investigated the relative abundance and identity of microbial contaminants of the edible long-horned grasshopper (*Ruspolia differens*) harvested from the wild and traded in informal markets in Uganda, to reveal high health risk points. Raw samples of whole *R. differens* were collected from wild vegetation, trapping sites and markets. Additionally, samples of plucked and deep-fried ready-to-eat *R. differens* were collected from the markets. The samples were cultured on standard media for microbial quantification, and pure cultures were characterised using molecular techniques. Bacterial and fungal counts in deep fried ready-to-eat *R. differens* were ~3- and 2-fold lower, respectively, than in raw samples. Loads of these microbes in deep fried insects were within recommended food safety limits. The highest bacterial counts were detected in whole *R. differens* samples from the market followed by trapping points. The fungal counts in the raw *R. differens* were comparable across the sampling points. The bacterial and fungal counts in *R. differens* in Kampala were not influenced by district of origin. Seven species of bacteria and seven species of fungi were recorded in *R. differens* samples. The microbial species were most diverse in samples from trapping points and least diverse in the deep-fried insects. The key pathogenic bacteria detected in marketed *R. differens* were *Staphylococcus sciuri*, *Acinetobacter baumannii* and *Serratia marcescens*, all of which were absent in wild-caught whole insects. Our results demonstrate that *R. differens* obtained at the trapping sites and markets are contaminated with potentially harmful microbes, therefore they require processing through deep frying to minimise health risks associated with their consumption. Further studies are warranted to elucidate specific handling practices at distribution and trapping points which may prevent introduction of microbes into *R. differens*.

Keywords: markets, food safety, food processing, *nsenene*, trapping sites

1. Introduction

Edible insects have gained global recognition in recent years as alternative sources of food and feed with a comparable or superior nutritional content than conventional animal products (Dobermann *et al.*, 2017; Kelemu *et al.*, 2015; Van Huis, *et al.*, 2013). The rich nutritional value of edible insects coupled with high moisture content provide a conducive environment for microbial contamination, survival and growth (Klunder *et al.*, 2012; Van Huis, *et al.*, 2013). Microorganisms can also be introduced along the food handling chain, causing food spoilage or disease

outbreaks (Ng'ang'a *et al.*, 2019; Ssepuuya *et al.*, 2019). Intrinsic factors (e.g. pH, moisture content and water activity) and extrinsic factors (e.g. temperature, relative humidity, number and type of microorganisms present) influence microbial survival and growth in foods (Charpe *et al.*, 2019; Garofalo *et al.*, 2019). The number and type of microorganisms in foods is highly influenced by the environment where the food originated and the sanitary conditions under which it is handled and processed (Belluco *et al.*, 2013; Van Huis *et al.*, 2013). Also, the symbiotic association of many insect species with some fungi and bacteria (Engels and Moran, 2013) enables them to thrive

and survive in the insects' gut (Colman *et al.*, 2012). This also possibly causes spoilage once the insect dies.

Several studies have highlighted the presence of different genera of bacteria and fungi in fresh and processed (ready-to-eat) insects e.g. the migratory locust, *Locusta migratoria* L (Orthoptera: Acrididae) and mealworm, *Tenebrio molitor* L (Coleoptera: Tenebrionidae) (Garofalo *et al.*, 2017; Stoops *et al.*, 2016); crickets, *Acheta domesticus* L and *Gryllobates sigillatus* (Walker) (Orthoptera: Gryllidae) (Osimani *et al.*, 2017; Vandeweyer *et al.*, 2017); and the long-horned grasshopper, *Ruspolia differens* (Serville) (Orthoptera: Tettigoniidae) (Ng'ang'a *et al.*, 2019; Ssepuuya *et al.*, 2019). Counts of total aerobic microbes, yeasts and moulds higher than the maximum standard limits set out in standards for edible insects (KEBS, 2020; Stoops *et al.*, 2016; UNBS, 2020) have been reported in wild harvested raw *R. differens* (Ng'ang'a *et al.*, 2019; Ssepuuya *et al.*, 2019).

In East Africa, the long-horned grasshopper, *R. differens* commonly called *nsenene* forms a major part of the food culture, constituting 5-10% protein intake of some populations (Ayieko *et al.*, 2012; Kinyuru *et al.*, 2010). This insect exists predominantly in two colour morphs, i.e. light brown and light green (Leonard *et al.*, 2020). Swarms of *R. differens* occur twice a year during rainy seasons (Agea *et al.*, 2008; Mmari *et al.*, 2017; Ssepuuya *et al.*, 2017). The insects are harvested at night using high light intensity fluorescent bulbs to attract them onto slanting silver iron sheets that slide the catch into harvesting drums (Okia *et al.*, 2017). Materials such as moist cassava flour are applied on the drum walls and iron sheets to make them slippery to prevent the insects from escaping. These materials may predispose the trapped insects to contamination by harmful microbes hence reducing their quality and increasing health risks to consumers. In Uganda, Masaka district is among the leading sites of harvesting *R. differens* while Kampala city is the hub of trade in these grasshoppers originating from different locations (Agea *et al.*, 2008; Ssepuuya *et al.*, 2017). These locations are therefore ideal for investigation of microbial contamination of *R. differens* along its value chain.

After harvesting, trappers manually transfer *R. differens* from the drums into aerated polythene/nylon/sisal bags. Most of the harvest is then sold to dealers who transport whole insects by public means to distribution points in other districts. In such points, the insects which are inactive due to anoxia are sold to retailers who then process them before selling to final consumers or other middle persons. Retailers with large quantities of raw *R. differens* kill and preserve some insects by freezing before processing. Processing of *R. differens* is usually carried out for value addition and to increase on shelf stability, but food processing methods may impact on safety and nutritional value (Bokulich *et al.*, 2016; Nyangena *et al.*, 2020). Processing *R. differens* begins with sorting out non-target insects and plucking (removal

of wings, antennae and legs) followed by washing with clean water. To speed up the cleaning process, materials such as wood ash, maize flour or cassava flour are employed to increase friction (Ssepuuya *et al.*, 2019). The plucked grasshoppers are usually deep fried (to increase the shelf life (Ssepuuya *et al.*, 2016)), cooled and sold ready-to-eat or stored in cardboard boxes. The involvement of several people during the post-harvest handling processes under different environmental conditions is likely to compromise *R. differens* quality and safety (Ssepuuya *et al.*, 2019). Therefore, this study aimed at quantifying and identifying bacterial and fungal contaminants at different points of the food handling chain for wild harvested *R. differens* in Uganda, to reveal high health risk points. The hypotheses tested were: (1) there is no difference in the loads and identity of bacteria and fungi in raw whole *R. differens* samples from wild vegetation, trapping sites and at markets, as well as processed (raw plucked and deep fried) samples from markets; (2) there is no difference in the loads of bacteria and fungi in *R. differens* from markets in Masaka and Kampala districts; and (3) loads of bacteria and fungi in *R. differens* distributed in Kampala are not influenced by their district of origin.

2. Materials and methods

Study area

This study was conducted in two purposively selected districts of Masaka and Kampala in central Uganda. From Masaka district, four *R. differens* trapping points (Kitaka, Nyendo-villa road, Kayirikiti A, Kayirikiti B) and three markets selling the insects (Nyendo, Masaka Municipal and Ssaza) were purposively sampled. Masaka district has a bi-modal rainfall pattern with peaks experienced between March to May and September to December. The mean annual rainfall in Masaka ranges between 1,100 and 1,200 mm with temperatures ranging between 18 and 28 °C (Masaka District Local Government, 2020). In Kampala district, *R. differens* was sampled from two distribution points (Katwe and Busega) and three markets (Kalerwe, Nakasero and Kasubi). Kampala district has a bimodal rainfall regime with peaks experienced in March to May and September to November, with mean annual rainfall ranging between 1,750 and 2,000 mm and temperatures of 17-28 °C (NEMA, 2014). During data collection, coordinates from sampling points were taken with a Global Positioning System (GPS) (GARMIN eTrex 20X, Garmin Ltd, Olathe, KS, USA) and plotted on Arc Map using the Arc GIS software version 10.3 (Esri Eastern Africa Ltd, Nairobi, Kenya) (Figure 1).

Sampling design and sample collection

R. differens samples weighing 200 g per batch were collected on 14 and 15 June (first season) and 23 and 24 November (second season) 2019 from each sampling point in Masaka

Figure 1. Locations of *Ruspolia differens* sampling sites in Masaka and Kampala, central Uganda in 2019.

and Kampala districts. In Masaka, a total of 36 batches were obtained in each season as follows: (1) 9 batches (3 replicates per site) of live whole *R. differens* collected from wild vegetation using a sweep net at Nyendo-vila road, Kayirikiti A and Kayirikiti B; (2) 9 batches (3 replicates per site) of raw whole *R. differens* randomly sampled from harvesting drums at Kayirikiti A, Nyendo-vila road and Kitaka trapping points; and (3) 9 batches of raw plucked and 9 batches of deep fried *R. differens* (3 replicates of each type) at Nyendo, Masaka Municipal and Ssaza markets. A total of 72 batches were therefore sampled in Masaka in the two seasons. In Kampala, 9 batches of raw whole *R. differens* from distribution points of Busega (3 replicates originating from Fort-portal district) and Katwe (3 replicates each from grasshoppers originating from Kasese and Kabale districts) were purchased in the second season only because samples collection in the first season commenced when supplies at the distribution points had become scarce. In addition, 18 batches of *R. differens* were purchased from Kampala in each season as follows: 9 batches of raw plucked and 9 batches of deep-fried *R. differens* (3 replicates of each type per market) from Nakasero, Kalerwe and Kasubi markets. In total, 27 batches of plucked and deep-fried *R. differens*

were obtained from Kampala in the two seasons. Each *R. differens* samples obtained was immediately placed in a sterile zip lock bag, kept in a cool box with flaked ice and transported to Makerere University, Department of Zoology, Entomology and Fisheries Sciences for preservation in Haier deep freezer HCF-300HTQ at -20 °C (Haier deep freezer, India) prior to transportation for microbial analysis at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi.

Microbiological analyses

From each batch of *R. differens* samples, 5 g were ground using a sterile pestle and mortar. These homogenates were resuspended in 45 ml buffered saline in 50 ml falcon tubes to form stock solutions. Serial dilution was done using 1 ml from the stock solution into another 9 ml phosphate-buffered saline in a 27 ml universal tube using a sterile pipette. The 10-fold dilution was repeated to obtain between 10^{-2} to 10^{-8} and 10^{-2} to 10^{-5} for isolation and analyses of bacteria and fungi, respectively (Ssepuuya *et al.*, 2019). Isolation and analyses of bacteria and fungi were done in triplicates on four different types of media

which included MacConkey agar and nutrient agar for bacteria, and potato dextrose agar and Sabouraud dextrose agar for fungi (Gatheru *et al.*, 2019). After 5 days, fungal colony counts were conducted with the aid of a dissecting microscope. Sub-culturing was done for plates with mixed colonies to obtain pure colonies. For bacteria, colony counts for individual plates were obtained after 48 hours. The counting was aided by a hand-held tally counter. The bacterial and fungal counts were computed and expressed as colony forming units per gram (cfu/g) according to Omoya and Akinyosoye (2013).

Biochemical characterisation

Pure bacterial isolates were classified by gram staining (Public Health England, 2019). For each isolate, a smear was prepared, fixed by heating gently and the slide flooded with 0.5% crystal violet dye. After 30 seconds, the slide was rinsed using water, flooded with 1% Lugol's iodine and covered with fresh iodine. The smear was then decolorised using 95% ethanol, rinsed with water and flooded with 0.1% counterstain safranin. The slide was washed, blot dried and examined using oil immersion objective under a total magnification of 1000× (Leica EZ4HD Stereo microscope, Leica Microsystems Inc., Heerbrugg, Switzerland). Pure cultures were later identified to species level using molecular techniques.

DNA extraction

Fungal colonies from pure cultures were harvested by scraping mycelia/spores using a sterile blade into 1.5 ml Eppendorf tubes, each containing three sterile beads and genomic DNA extracted using the plant DNA extraction kit (Bioline, London, UK) following the manufacturer's protocol. On the other hand, pure bacterial isolates were aseptically inoculated into 7 ml Luria broth in sterile 50 ml Falcon tubes placed into an Innova 44 incubator shaker (Eppendorf, Hamburg, Germany) set at 180 rpm and 37 °C for 18 hours. The cells were then harvested by centrifugation at 2,000 relative centrifugal force for 3 min into 1.5 ml Eppendorf tubes. Bacterial DNA was then extracted using the isolate II Genomic DNA Kit (Bioline) following the manufacturer's protocol.

PCR-amplification

Polymerase chain reaction (PCR) amplification of fungi was carried out as described by Gatheru *et al.* (2019). Briefly, all PCR reactions were carried out in a final 20 µl volume containing 5× MyTaq reaction buffer (Bioline) (5 mM dNTPs, 15 mM MgCl₂ stabilisers and enhancers), 0.5 pmol/µl of each primer (ITS5 (5' TCCTCCGCTTGATATTGATATGC 3') and ITS4 (5' GGAAGTAAAAGTCGTAACAAGG 3') (White *et al.*, 1990)), 0.25 mM MgCl₂, 0.0625 U/µl MyTaq DNA polymerase (Bioline) and 15 ng/µl of DNA template.

PCR reactions were set up in a mastercycler Nexers thermal cycler (Eppendorf). The cycling conditions involved an initial denaturation step at 95 °C for 10 min, 35 cycles of denaturation step at 94 °C for 1 min, an annealing step of 54 °C for 1 min and an extension step at 72 °C for 1 min followed by a final extension at 72 °C for 10 min (Gusmão *et al.*, 2007). The expected product size ranged between 450-600 bp.

To target the bacterial small sub-unit rRNA genes from the V1 to the V2 hyper variable region, the PCR amplification for bacteria was carried out using 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACCTTGTTACGACGACTT 3') primers (Rinttilä *et al.*, 2004). The PCR reactions set up was as described above except for the annealing temperature at 52 °C. The expected product size was approximately 1,500 bp. The amplified PCR products were resolved through a 1.2% agarose gel. Bands of DNA on the gel were analysed using KETA GL imaging system trans illuminator (Wealtec Corp, Meadowvale Way Sparks, NV, USA).

Sequencing of DNA and species identification

Successfully amplified products were sent to Macrogen Inc Europe Laboratory, Amsterdam, the Netherlands, for bi-directional sequencing. Bacteria and fungi were then identified by assembling sequences using BioEdit Sequence Alignment Editor Version 7.2.5 then multiple alignment done in Clustal X version 2.1 (Thompson *et al.*, 1997). The sequences were queried via Basic Local Alignment Search Tool (BLAST) at the GenBank database National Centre for Biotechnology Information (NCBI, 2015) for final identification. Sequences of identified microbes were deposited in GenBank (Supplementary Table S1).

Data analysis

Data on bacterial and fungal loads, expressed as log cfu/g, were tested for normality by plotting normal distribution curves and determining the homogeneity of variance. Because the data were normally distributed, one-way analysis of variance with 95% confidence interval was performed to test for significant differences in microbial counts at different sampling points. The sampling points considered were whole insects from the wild vegetation, trapping sites and markets; and plucked and deep-fried insects from markets. For whole *R. differens* marketed in Kampala, differences in microbial loads in samples originating from Kabale, Fort portal and Kasese districts were determined. Means were separated using Bonferroni adjustment. A two-sample t-test was used to determine if bacterial and fungal counts were significantly different between samples from Kampala and Masaka. All analyses were carried out in R-statistical computer program version 4.0.0 (R Development Core Team, 2019) at $\alpha=0.05$.

3. Results

Bacterial and fungal loads in *Ruspolia differens* from different sampling points

The mean bacterial counts in *R. differens* samples varied significantly with sampling points in both the first ($F_{2,43}=91,027$, $P<0.001$) and second ($F_{4,46}=33,954$, $P<0.001$) swarming seasons of 2019. The bacterial counts in deep fried *R. differens* were significantly lower than those in raw samples from all other sampling points in both seasons (Figure 2A). The bacterial counts in whole *R. differens* samples from the market in the second season were significantly higher than those in plucked samples in the market, and whole samples from trapping points and the wild vegetation.

There were also significant differences in fungal counts in *R. differens* from different sampling points in both swarming seasons ($F_{4,44}=5,973$, $P<0.001$ and $F_{4,46}=3,509$, $P<0.001$ for first and second seasons, respectively). The fungal counts in fried *R. differens* were significantly lower than those in raw samples from all sampling points (Figure 2B). There were however no significant differences in fungal counts in raw *R. differens* samples from all sampling points in both seasons.

Bacterial and fungal loads in *Ruspolia differens* samples from Kampala and Masaka

In the first season, the mean bacterial counts in *R. differens* samples from Masaka were significantly higher than those in samples from Kampala ($t=7.3$, $P<0.001$); Figure 3A). However, there was no significant difference in bacterial

counts in *R. differens* samples between the two districts in the second season ($t=0.29$, $P=0.83$). On the other hand, the mean fungal counts in *R. differens* samples were not significantly different between Masaka and Kampala in both first and second swarming seasons ($t=1.06$, $P=0.63$ and $t=1.32$, $P=0.12$ respectively; Figure 3B).

Bacterial and fungal loads in whole *Ruspolia differens* samples from distribution points in Kampala

Samples of whole *R. differens* from distribution points in Kampala were found to originate from Fort-portal, Kasese and Kabale districts. There were no significant differences in bacterial counts in whole *R. differens* samples from the distribution points based on the district of origin ($F_{2,6}=1.81$, $P=0.24$; ~ 8.1 log cfu/g for all samples). Similarly, there were no significant difference in fungal counts in whole *R. differens* samples from the distribution points ($F_{2,6}=0.63$, $P=0.56$; ~ 5.9 cfu/g for all samples).

Species characterisation of fungi and bacteria in *Ruspolia differens* samples

A total of 49 sequences were matched for identity with sequences of the data base and all showed an identity similarity of between 90 and 100% to the sequences in the GeneBank. Thirty of these sequences originated from pure fungal isolates while 19 came from pure bacterial isolates. From the isolates, 7 species each of bacteria and fungi were recorded (Figure 4; Supplementary Table S1). There were variations in the distribution of microbial species and strains at different sampling points. The highest numbers of bacterial (05) and fungal (04) species were recorded in

Figure 2. Mean (A) bacterial and (B) fungal counts in *Ruspolia differens* from different sampling points in Uganda in the first and second seasons of 2019. Error bars represent standard errors of the mean.

Figure 3. Mean (A) bacterial and (B) fungal counts in *Ruspolia differens* samples from Masaka and Kampala in the first and second seasons of 2019. Error bars represent standard errors of the mean.

R. differens samples from harvesting drums at trapping sites and the lowest (01 species for both) were recorded in fried ready-to-eat insects from the markets.

Only one bacterial species (*Bacillus cereus* with 6 strains) was recorded at all sampling points. *Staphylococcus* sp. (1 strain) and *Bacillus thuringiensis* (2 strains) were only recorded in harvesting drums at trapping sites, *Acinetobacter baumannii* at distribution points, whereas *Roseomonas* sp. (1 strain) was recorded only in plucked samples sold at markets. The fungal species *Trichoderma asperellum* (with 9 strains) was recorded at all sampling points; while *Papiliotrema laurentii* (1 strain) was recorded only in whole samples from the wild vegetation. Most species recorded in samples from trapping sites and distribution points or markets were not recorded from insects collected from the wild vegetation (Figure 4). The pathogenic bacterium *B. cereus* and the fungus *T. asperellum* were recorded in fried ready-to-eat insects and in plucked samples sold at markets.

4. Discussion

High levels of bacterial and fungal contaminants have been previously recorded in wild-harvested edible *R. differens* (Ng'ang'a *et al.*, 2019; Ssepuuya *et al.*, 2019). However, prior to this study, levels of these microbes at different points of *R. differens* handling chain in Uganda had not yet been determined to identify high health risk points. The loads and identity of bacteria and fungi in raw samples of whole *R. differens* from the wild vegetation, trapping sites and at markets, as well as processed (plucked and deep fried) samples from markets during two swarming seasons in 2019 at Masaka and Kampala districts, central Uganda were determined in this study.

Our data show that bacterial counts were significantly higher in whole *R. differens* samples from distribution

points than in plucked samples in the markets, and whole samples from the trapping points and the wild vegetation. Potential sources of contamination of *R. differens* at the distribution points are market environment, confinement in bags and human handling. Oranusi and Braide (2012) and Musundire *et al.* (2016) reported that primitive and unhygienic methods of handling, packaging and storage of edible insects cause contamination with microbes. The significant decline in bacterial counts in raw *R. differens* after plucking in the market could be attributed to extraneous contamination from the environments and handlers, which could most likely settle on appendages which are removed during plucking.

Unlike bacteria, the fungal counts in raw *R. differens* at different sampling points were not statistically different. Fungal growth turn-over rates take hundreds of days unlike bacteria with turn-over rates of hours to few days (Rousk and Bååth, 2011). Therefore, it is unlikely for the fungal populations to change drastically from the time *R. differens* is moved from the wild vegetation to the traps, to the time they are plucked in the market, which takes 1-2 days. The use of relative abundance of colony forming units along *R. differens* handling chain may therefore not suffice in determining high risk points for fungal contaminants.

The bacterial and fungal counts in deep fried ready-to-eat *R. differens* were 3- and 2-fold lower, respectively, than in raw insects at all sampling points. This could be attributed to the efficiency of deep frying in reducing microbial loads in edible insects (Gatheru *et al.*, 2019). The deep fried *R. differens* had lower bacterial and fungal counts (approximately 3 log cfu/g) than the maximum limits of 5 log cfu/g and 2-3 log cfu/g set out in the standards on edible insect products in Uganda and Kenya (KEBS, 2020; UNBS, 2020). These maximum limits are comparable with those of minced meat which are recommended for use in

Contaminant microbe	Whole insect			Processed insect in the market	
	Wild vegetation	Harvesting drums	Market	Plucked	Deep-fried
Gram positive bacteria					
<i>Bacillus cereus</i>					
<i>Bacillus thuringiensis</i>					
<i>Staphylococcus</i> sp. strain					
<i>Staphylococcus sciuri</i>					
Gram negative bacteria					
<i>Serratia marcescens</i>					
<i>Acinetobacter baumannii</i>					
<i>Roseomonas</i> sp.					
Basidiomycota					
<i>Rhodotorula muscilaginosa</i>					
<i>Rhodotorula dairenensis</i>					
<i>Trichosporon asahii</i>					
<i>Papillotrema laurentii</i>					
Ascomycota					
<i>Trichoderma asperellum</i>					
<i>Clavospora lusitanae</i>					
<i>Pichia kudriavzevii</i>					

Figure 4. Bacterial and fungal species isolated from *Ruspolia differens* at different sampling points.

edible insects in many parts of the world (FAO/WHO, 2002; Ssepuuya *et al.*, 2019; Stoops *et al.*, 2016; UNBS, 2019). Despite the low bacterial and fungal counts in deep fried ready-to-eat *R. differens* sold in Ugandan markets, the presence of *B. cereus* may compromise its safety for human consumption. Efforts aimed at eliminating/ preventing re-contamination of deep fried ready-to-eat *R. differens* with these microbes are therefore necessary for their safety. Further research is also required to examine the effect of other factors like packaging procedure, duration of sale and hygienic practices of handlers on bacterial and fungal loads in deep fried *R. differens*. On the other hand, the bacterial and fungal counts in raw *R. differens* were higher than the limits recommended for minced meat suggesting that the consumption of raw *R. differens* as practiced by some people in Uganda (Agea *et al.*, 2008), is unsafe. This could be attributed to the high nutrient and moisture content in *R. differens* (Kinyuru *et al.*, 2010), which provides a conducive atmosphere for microbial contamination, growth and survival (Klunder *et al.*, 2012).

There was no detectable difference in fungal counts in *R. differens* collected from Masaka and Kampala during both swarming seasons. However, the bacterial counts in samples from Masaka were significantly higher than in those from Kampala during the first season only. The first rainy season in Uganda is normally wetter than the

second season (Nsubuga *et al.*, 2014), which may account for the different results in the two seasons. This finding partly corroborates the report by Bagumire and Karumuna (2017) that microbial loads in ready-to-eat meat sold along high ways in Uganda during the wet season were higher than in the dry season. The specific reasons for the higher bacterial counts in *R. differens* samples from Masaka than those from Kampala in the first season remain unknown.

This study further established that the bacterial and fungal counts in whole *R. differens* at distribution points in Kampala were not influenced by their district of origin. Ssepuuya *et al.* (2019) reported that *R. differens* traders in Uganda normally buy and package freshly harvested insects in bags and transport them by public means to distribution points. The similarity in packaging, storage and transportation practices across traders may account for the observed similar level of bacterial and fungal contamination of raw whole *R. differens*, irrespective of the district of origin.

Marked variation in number of bacterial and fungal species and/or strains were recorded at different *R. differens* sampling points. This could be attributed to adaptive dynamic shifts in response to changes in niche/community composition (Levy and Borenstein, 2013). The pathogenic bacteria (*Staphylococcus* sp., *Staphylococcus sciuri*, *B. thuringiensis* and *Serratia marcescens*) and fungus (*Rhodotorula dairenensis*)

were recorded in *R. differens* samples from the harvesting drums and not in samples from the wild vegetation. This could be attributed to unhygienic practices during trapping such as smearing drum walls and iron sheets with moist cassava flour to prevent escape of the catch (Okia *et al.*, 2017). Cassava flour processed under unhygienic conditions could be contaminated with microbes including those that produce mycotoxins (Kaaya and Eboku, 2010; Omara *et al.*, 2020). Furthermore, some bacteria such as *Staphylococcus* spp. and *Bacillus* spp. have been recorded in dry cassava flour (Ogori and Gana, 2013). Therefore, application of unhygienic moist cassava flour into *R. differens* harvesting drums may be an important entry point for these microbes. *Bacillus* spp. have also been detected in other edible insects (e.g. *Oryctes monoceros* (Banjo *et al.*, 2006), *Scapsipedus icipe* (Gatheru *et al.*, 2017) and *T. molitor* (Klunder *et al.*, 2012)). *Bacillus* spp. are a threat to human health and are known to cause food spoilage (Klunder *et al.*, 2012; Stoops *et al.*, 2016; Van Huis *et al.*, 2013). *S. marcescens* – an opportunistic pathogen (Khanna *et al.*, 2013), may also probably gain entry into *R. differens* through injuries sustained when they struggle to escape from harvesting drums or from manual handling by humans. Other pathogens possibly on non-target species trapped with *R. differens* (Sengendo *et al.*, 2021), could also be additional sources of microbial contamination. This could further explain the relatively high number of bacterial species recorded in samples from the harvesting drums than from the wild vegetation.

The bacterium *B. cereus* present at all *R. differens* sampling points is associated with many insect taxa, aiding their survival by synthesising essential nutrients (Khanna *et al.*, 2013). *R. differens* trapped with other by-catches could have been contaminated by insects containing these microbes and possibly upon contact with soil. The presence of *B. cereus* in deep fried ready-to-eat *R. differens* could be attributed to their ubiquitous nature (Podschnun *et al.*, 2001) coupled with unhygienic practices which predispose the insects to such contaminants. Ssepuuya *et al.* (2016) reported that *R. differens* vended in transparent polythene bags/plastic buckets were often left open to attract buyers, which exposes the insects to environmental contaminants. Klunder *et al.* (2012) noted that some *Bacillus* spp. are resistant to heat treatment and hence could be found in ready-to-eat-foods, causing food spoilage or health problems to consumers.

The presence of *A. baumannii* and *Trichosporon asahii* in samples from distribution points but not in samples from the wild vegetation and harvesting drums suggests that they could have been picked up during transportation. Also, *Roseomonas* sp. recorded in plucked samples only suggests that it could have been introduced during plucking, possibly upon contact with contaminated soil/water (Sandoe and Malnick, 1997). The risk of food contamination with

pathogens increases with a long chain of food handlers (Woh *et al.*, 2017).

The pathogenic bacteria *S. marcescens* and *Staphylococcus* spp. were detected in *R. differens* from all sampling points except in wild-collected insects. This could be attributed to contamination arising from handling by different people (some of whom may not practice proper personal hygiene) at the different points of the food handling chain (Banjo *et al.*, 2006).

As much as the bacterial and fungal counts in deep fried *R. differens* were below the maximum standard limits of minced meat, the presence of the foodborne pathogen *B. cereus*, *S. marcescens* and *Staphylococcus* spp. is a safety concern that needs to be addressed. Moreover, the vending practices of these foods in open buckets/polythene bags to attract buyers exposes them to environmental contaminants which reduces on their shelf life and causes health risks to consumers (Ssepuuya *et al.*, 2016). Further research for economically feasible innovations of packaging and storing *R. differens* traded in markets/streets, as well as strict regulations on handling the insects are required.

Only one fungal species, *P. laurentii* was isolated exclusively from wild-caught *R. differens*. *P. laurentii* is ubiquitous and prevalent in the soil rhizosphere, where it assimilates a wide range of hexoses, pentoses and organic acids (Leguina *et al.*, 2019). The natural interaction of *P. laurentii* with *R. differens* requires further investigation.

5. Conclusions

This study has revealed that bacterial and fungal counts in deep-fried ready-to-eat *R. differens* sold in Uganda were markedly lower than in raw insects and the former are within recommended food safety limits. The highest total bacterial counts were detected in whole samples in the markets followed by whole samples collected from the harvesting drums. The fungal counts in raw *R. differens* were comparable at all sampling points. The bacterial counts in *R. differens* were higher in Masaka than Kampala in the first season only. The bacterial and fungal counts in *R. differens* in Kampala were not influenced by district of origin. Seven species of bacteria and seven species of fungi were recorded in *R. differens* samples. The microbial species were most diverse in samples from trapping points and least diverse in the deep-fried insects. The key foodborne bacterial pathogens detected in *R. differens* samples were *B. cereus*, *S. sciuri*, *A. baumannii*, *Staphylococcus* sp. strain and *S. marcescens*, of which only *B. cereus* was present in wild-caught whole insect samples. These findings suggest that distribution and trapping points are hotspots for contamination of *R. differens* with microbes, and that processing the insects by plucking and deep frying significantly reduces microbial contamination. Further

studies are warranted to elucidate specific handling practices at distribution and trapping points which may prevent introduction of microbes into *R. differens*.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/JIFF2020.0069>.

Table S1. Diversity of microbes isolated from *Ruspolia differens* at different sampling points in Uganda in 2019.

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